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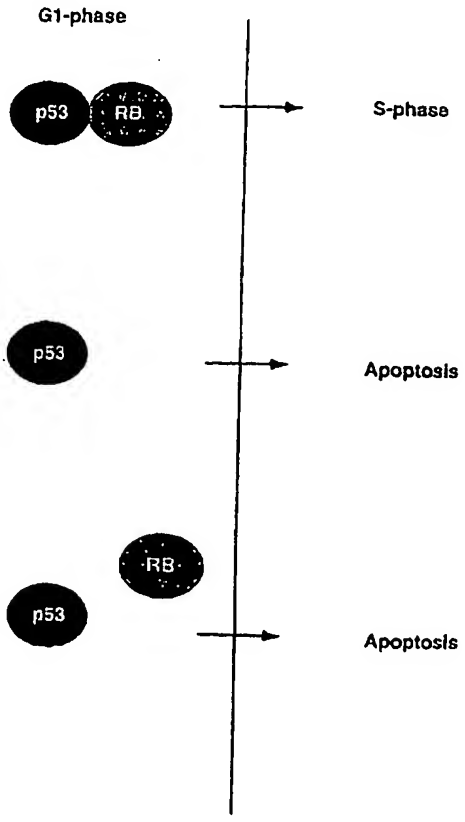
(54) Title: METHOD AND MEANS FOR DISRUPTION OF p53 AND RB INTERACTION

(57) Abstract

p53 mediates induction of apoptosis in RB mutant cells. p53 and RB interact at identified binding sites and interference with such interaction may be used in induction of apoptosis, useful for example in anti-tumor therapy, and modulation of other p53-mediated activities and properties of p53. Inhibitors or p53/RB interaction include peptide fragments of p53 and variants thereof, and non-peptidyl analogues and mimetics thereof. Assay methods and means allow for screening for inhibitor compounds which may induce apoptosis or modulate other p53-mediated activities or properties or p53 and be useful in therapeutic contexts.



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METHODS AND MEANS FOR DISRUPTION OF P53 AND RB INTERACTION

The present invention relates to screening methods, peptides, mimetics, and methods of use based on the surprising discovery and characterisation of an interaction between two known proteins and the recognition that this interaction plays a key role in whether or not a cell enters metaphase or whether apoptosis is induced. The proteins in question are p53 and RB (product of the Retinoblastoma gene). By interfering with the interaction, the balance can be shifted in favour of apoptosis. This gives rise to screening methods for identifying compounds which interfere with this interaction and which may induce apoptosis or modulate another p53-mediated cellular activity or function, useful in a therapeutic context, for example in the treatment of tumours or other malignancies. Furthermore it gives rise to the rational design of peptides or mimetics or functional analogues which fulfil this function.

The p53 protein plays a key role in DNA-damage induced cell cycle arrest Keubitz, S.J., et al. Proc Natl Acad Sci USA 1992 89 7491-7495 and apoptosis Clarke, A.R., et al., Nature 1993 362, 849-852 and Lowe, S.W. et al., Nature, 1993, 362, 847-849. Other roles of p53 are discussed below. The p53 gene is found to be mutated in a large proportion of tumours Hollsten, M. et al. Science 1991, 253, 49-53 suggesting that its inactivation is a key target in

carcinogenesis.

Animal models have shown that loss of p53-induced apoptosis is an important event in tumour progression Symonds, H. et al., Cell, 1994, 703-711. This suggests that mutations in the p53 gene occur after other oncogenic changes. Consistent with this model is the fact that p53 mutations are associated with late stages of tumour progression in certain cancers Fearon, E.R., et al., Cell, 1990, 61, 759-767 and are indications of poorer prognosis in a number of malignancies Fisher, D.E., Cell, 1994, 78, 539-542.

The Retinoblastoma (RB) protein is also known as a tumour suppressor protein (Lee *et al.* (1987) *Science* 235: 1394-9; Friend *et al.* (1987) *PNAS USA* 84: 9059-63). Furthermore, the ability of p53 to induce apoptosis correlates well with the absence or inactivation of the RB protein Morgenbesser, S.D. et al., Nature 1994, 371, 72-74. The inability of p53 to induce apoptosis in the presence of functional RB is supported by experiments showing that RB acts positively to inhibit apoptosis by ionising radiation Haas-Kogan, D. et al., EMBO J, 1995, 461-472.

The present invention in various aspects is founded on the surprising discovery that the p53 protein can contact the product of the Retinoblastoma gene. This interaction has been demonstrated experimentally both *in vitro* using various techniques,

and *in vivo*. p53 interacts via residues within its N-terminal activation domain (residues 1-71, within residues 1-73). Using *in vitro* translated p53 and GST-RB, it has been shown that p53 interacts with residues with positions 379-928 of RB. A C-terminal portion of p53, within residues 290-393, has also been demonstrated experimentally to interact with RB, as described below.

In addition, it has now been found that the activation domain of p53 (residues 1-71)has sequence similarity with the activation domain of the E2F transcription factor (Martin *et al.* (1995) *Nature* 375 691). This alignment indicates that the conservation overlaps the residues in E2F that are sufficient for the binding of RB (E2F residues 409-426, Helin, K. et al., Cell 1992, 70, 337-350. Point mutagenesis of E2F has shown which residues are required for RB binding Hagemeier, C., et al. Nucleic Acids Research 1993, 21, 4998-5004 and it has now been found that there are homologous residues in p53, providing insight into the interaction with RB and useful information for the design of molecules such as peptides which interfere with p53/RB interaction. A further region of p53 homologoues to E2F is found within the C-terminal portion of p53 found to bind RB.

The sequence similarity between p53 and E2F also reflects the binding sites for two other proteins: the TAT-box binding protein, TBP, and the MDM2 oncoprotein.

The fact that MDM2 and TBP bind to homologous sequences in p53 and E2F supports the conclusion that the sequence alignment reflects protein binding sites. It appears therefore that the RB binding residues in p53 are in an analogous position (in the alignment) with the RB binding residues in E2F.

The fact that p53 is unable to induce apoptosis in the presence of RB suggests in the light of the present invention that the binding of RB and p53 arrests p53-mediated apoptosis either directly or indirectly. Disruption of the p53-RB interaction is therefore indicated as a therapeutic target. For instance, by generating agents which disrupt the interaction of p53 with RB, and introducing such molecular agents into cancer or other tumour cells, or other cells for which it would be advantagous to induce cell death, specific cellular self destruction via p53-mediated apoptosis may be induced, for instance virally-infected cells (which may display a viral peptide facilitating targeting).

Evidence is provided herein indicating a role for binding of RB and p53 in protecting against p53-induced apoptosis, in the transcriptonal repressor function of p53 and stabilisation of p53 protein. Mutagenesis of RB binding residues of p53 affects the ability of p53 to induce apoptosis, its ability to repress transcription, a function which also correlates wih

apoptosis, and stabilisation of p53 protein. Details of the experiments which have been performed and further discussion of the role of p53 and its interaction with RB are included below.

The present invention in various aspects provides for interfering with or interrupting interacion between the Retinoblastoma protein (RB) and p53, using an appropriate agent.

Such an agent may be capable of blocking binding between a site located at amino acid residues 43-60 on mouse p53 (see Figure 3A) and a site on RB; a site located at residues 40-50 of human p53 (see Figure 3B) and a site on RB; a site locatedd at residues 11-29 in human p53 (see Figure 3B) and a site on RB; or a site located within residues 290-393 of human p53 (e.g. 370-393; see Figures 3B and 3C). The full sequence of the p53 protein has been elucidated and is set out in WO93/20238 which is incorporated herein by reference and in Figure 3C. Relevant portions of p53 are shown in Figure 3A (mouse) and Figure 3B (human) and the complete amino acid and encoding nucleic acid sequences for human p53 appear in Figure 3C (including the C-terminal portion which includes an additional RB binding site). The RB sequence is given in Lee *et al.* and Friend *et al.* (*supra*). The numbering of the amino acids used herein for p53 corresponds with that set out

in WO93/20238.

Such agents may be identified by screening techniques which involve determining whether an agent under test inhibits or disrupts the binding or RB protein or a suitable fragment thereof, with a fragment of p53 which includes amino acid residues 43-60 of the mouse, amino acids 40-50 or 40-45 of the human, or 11-29 of the human or within 290-393, e.g. 370-393, of the human, or a suitable analogue, fragment or variant thereof.

Suitable fragments of RB include those which include residues 379-928. Smaller fragments, and analogues and variants of this fragment may similarly be employed, e.g. as identified using a technique such as alanine scanning.

Screening methods and assays are discussed in further detail below.

p53 appears to be a sensor for damaged DNA. when such DNA damage occurs during a stage of the cell cycle when it can be repaired, p53 induces arrest in the G1 phase. Following the repair of DNA the cell continues its cycle. If, however, this damage is not likely to be repaired before the cell replicates its DNA, p53 induces apoptosis. Thus, p53 can induce G1 arrest or apoptosis (Kouzarides, T. (1995), *Trends in Cell Biol.*, Vol. 5, pp448-450). It is known that RB prevents p53-mediated apoptosis. RB may also affect p53-mediated G1

arrest by binding its binding to p53.

p53 may also affect homologous recombination, since it binds to Rad51, a protein involved in this process (Stürzbecher, H.-W., *et al.* (1996), *EMBO*, Vol. 15, No. 8, pp1992-2002). It may affect DNA repair processes directly since it binds TFIIH, a protein involved in this process (Léveillard, T., *et al.* (1996), *EMBO*, Vol 15, No. 7, pp1615-1624). p53 is also reported to contain 3' to 5' exonuclease activity (Mummenbrauer, T., *et al.* (1996), *Cell*, Vol. 85, pp1089-1099). These functions of p53 may be involved in guarding the genome from damage. In view of the present invention it can be seen that RB may regulate these activities and functions and others mediated by p53.

Thus, various methods and uses of inhibitors of p53 and RB interaction are provided as further aspects of the present invention. The purpose of disruption, interference with or modulation of interaction between p53 and RB may be to modulate any p53-mediated activity, such as apoptosis, transcriptional repression, G1 arrest, DNA repair, homologous recombination, and so on, or to modulate an activity of p53, such as 3' to 5' exonuclease activity.

"TBP" (TATA-binding protein, the DNA-binding subunit of transcription factor IID (TFIID) (Horikoshi, N. *et al.* (1995), *Mol. and Cell Biol.*, Vol. 15, No. 1,

pp237-234and references cited therein) binds the N-terminus of p53 (residues 20-57) and the C-terminus (residues 220-271). These binding sites overlap with the regions shown herein to bind with RB. Furthermore, the deletion of p53 residues 40-45 is shown to affect both RB and TBP binding. Binding of TBP to p53 may be involved in mediation of repression of transcription by p53 and also its mediation of apoptosis.

The MDM2 binding site on p53 (residues 22/23) is also shown herein to be a binding site for RB. It has been shown that MDM2 inhibits the G1 arrest and apoptosis functions of p53 (Chen, J., *et al.* (1996), *Mol. and Cell Biol.*, Vol. 16, No. 5, pp2445-2452), so RB may be opposing such action of MDM2.

Experimental evidence included below provides indication that mutation within the MDM2 binding site of p53 stabilises p53, i.e. extends its half-life. Thus, a further purpose of using an inhibitor of p53/RB binding in accordance with various aspects of the present invention may be to stabilise p53.

An inhibitor of binding of RB to p53 employed in accordance with various aspects of the present invention may or may not also inhibit binding to p53 of TBP, MDM2 and/or any other molecule which binds in one or more of the regions of p53 bound by RB. In certain embodiments, it may be preferable to employ an inhibitor of p53/RB binding which does not inhibit TBP,

MDM2 or other molecule binding to p53, for example where that molecule is involved in mediation of a p53 function or activity, such as apoptosis. Such discriminatory inhibitors may be identified using screening assays as disclosed below, further including within the test the relevant molecule such as TBP and/or MDM2 and further determining the binding of the molecule to p53.

One class of agents that can be used to disrupt the binding of p53 and RB are peptides based on the sequence motifs of p53 that interact with RB. Such peptides tend to be small molecules, and may be about 40 amino acids in length or less, preferably about 35 amino acids in length or less, more preferably about 30 amino acids in length, or less, more preferably about 25 amino acids or less, more preferably about 20 amino acids or less, more preferably about 15 amino acids or less, more preferably about 10 amino acids or less, and more preferably about 5 amino acids or less in length. The present invention also encompasses peptides which are sequence variants or derivatives of a wild type p53 sequence.

The p53 employed may be human or mouse or of any other suitable species.

Instead of using a wild-type p53 fragment, a peptide or polypeptide may include an amino acid sequence which differs by one or more amino acid

residues from the wild-type amino acid sequence, by one or more of addition, insertion, deletion and substitution of one or more amino acids. Thus, variants, derivatives, alleles, mutants and homologues, e.g. from other organisms, are included.

Preferably, the amino acid sequence shares homology with a fragment of the relevant p53 fragment sequence shown in Figure 3A, 3B or 3C, preferably at least about 30%, or 40%, or 50%, or 60%, or 70%, or 75%, or 80%, or 85% homology, or at least about 90% or 95% homology.

As is well-understood, homology at the amino acid level is generally in terms of amino acid similarity or identity. Similarity allows for "conservative variation", i.e. substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine. Similarity may be as defined and determined by the TBLASTN program, of Altschul *et al.* (1990) *J. Mol. Biol.* 215: 403-10, which is in standard use in the art. Homology may be over the full-length of the relevant peptide or over a contiguous sequence of about 5, 10, 15, 20, 25, 30 or 35 amino acids, compared with the relevant wild-type amino acid sequence.

A peptide fragment of p53 which may be employed to inhibit p53/RB interaction may include the sequence:

D D L L L P Q D V E E F F E G P S E

corresponding to residues 43-60 of mouse p53, or

M D D L M L S P D D,

corresponding to residues 40-50 of human p53, or

E P P L S Q E T F S D L W K L I P E N,

corresponding to residues 11-29 of human p53, or

K S K K G Q S T S R H K K L M F K T E G P D S D,

corresponding to residues 370-393 of human p53, or other portion of the C-terminal residues 290-393 of p53.

As noted, variant peptide sequences and peptide and non-peptide analogues and mimetics may be employed, as discussed further below.

Various aspects of the present invention provide a substance, which may be a single molecule or a composition including two or more components, which includes a peptide fragment of p53 which includes a sequence as recited above and/or disclosed elsewhere hrein, a peptide consisting essentially of such a sequence, a peptide including a variant or analogue sequence, or a non-peptide analogue or mimetic which has the ability to bind RB and/or disrupt or interfere with interaction between p53 and RB.

Suitable analogues of p53 which may be employed in the present invention include peptides which include the following sequence (where "-" indicates the

presence of any amino acid:

- D L - L - - D - - E - F - - - - E

and which function to bind RB protein and/or disrupt binding between p53 and RB. The residues specified in this peptide are indicated as important for RB binding based on homology of mouse p53 with the E2F RB binding site (Figure 3A).

Variants include peptides in which individual amino acids can be substituted by other amino acids which are closely related as is understood in the art and indicated above. For example, any of the above mentioned residues could be substituted by E; L residues may be substituted by F or Y; and I residues substituted by F.

One particular variant of this, based on sequences conserved in mouse and human p53 and E2F1, includes

- D L - L - - D - - E

and may include one or more additional amino acids, as discussed.

The following shows a line-up of residues in the region of an RB binding site in E2F1, and mouse p53 and human p53 peptides in accordance with various embodiments of the present invention:

E2F1	A L D Y H F G L E E G E G I R D L F D
Mouse p53	D D L L L P Q D V E E F F E G P S E
(43-60)	- D L - L - - D - - E - F - - - - E
Human p53	
(40-50)	M D D L M L S P D D I E
	- D L - L - - D - - E

See also Figures 3A and 3B.

Non-peptide mimetics of peptides are discussed further below.

As noted, a peptide according to the present invention and for use in various aspects of the present invention may include or consist essentially of a fragment of p53, such as a fragment whose sequence is given above. Where one or more additional amino acids are included, such amino acids may be from p53 or may be heterologous or foreign to p53. A peptide may also be included within a larger fusion protein, particularly where the peptide is fused to a non-p53 (i.e. heterologous or foreign) sequence, such as a polypeptide or protein domain.

The invention also includes derivatives of the peptides, including the peptide linked to a coupling partner, e.g. an effector molecule, a label, a drug, a toxin and/or a carrier or transport molecule.

Techniques for coupling the peptides of the invention to both peptidyl and non-peptidyl coupling partners are well known in the art. In one embodiment, the carrier molecule is a 16 aa peptide sequence derived from the homeodomain of *Antennapedia* (e.g. as sold under the name "Penetratin"), which can be coupled to a peptide via a terminal Cys residue. The "Penetratin" molecule and its properties are described in WO 91/18981.

Peptides may be generated wholly or partly by chemical synthesis. The compounds of the present invention can be readily prepared according to well-established, standard liquid or, preferably, solid-phase peptide synthesis methods, general descriptions of which are broadly available (see, for example, in J.M. Stewart and J.D. Young, Solid Phase Peptide Synthesis, 2nd edition, Pierce Chemical Company, Rockford, Illinois (1984), in M. Bodanzsky and A. Bodanzsky, The Practice of Peptide Synthesis, Springer Verlag, New York (1984); and Applied Biosystems 430A Users Manual, ABI Inc., Foster City, California), or they may be prepared in solution, by the liquid phase method or by any combination of solid-phase, liquid phase and solution chemistry, e.g. by first completing the respective peptide portion and then, if desired and appropriate, after removal of any protecting groups being present, by introduction of the residue X by reaction of the respective carbonic or sulfonic acid or

a reactive derivative thereof.

Another convenient way of producing a peptidyl molecule according to the present invention (peptide or polypeptide) is to express nucleic acid encoding it, by use of nucleic acid in an expression system.

Accordingly the present invention also provides in various aspects nucleic acid encoding the polypeptides and peptides of the invention.

Generally, nucleic acid according to the present invention is provided as an isolate, in isolated and/or purified form, or free or substantially free of material with which it is naturally associated, such as free or substantially free of nucleic acid flanking the gene in the human genome, except possibly one or more regulatory sequence(s) for expression. Nucleic acid may be wholly or partially synthetic and may include genomic DNA, cDNA or RNA. Where nucleic acid according to the invention includes RNA, reference to the sequence shown should be construed as reference to the RNA equivalent, with U substituted for T.

Nucleic acid sequences encoding a polypeptide or peptide in accordance with the present invention can be readily prepared by the skilled person using the information and references contained herein and techniques known in the art (for example, see Sambrook, Fritsch and Maniatis, "Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989, and

Ausubel et al, Short Protocols in Molecular Biology, John Wiley and Sons, 1992), given the nucleic acid sequence and clones available. These techniques include (i) the use of the polymerase chain reaction (PCR) to amplify samples of such nucleic acid, e.g. from genomic sources, (ii) chemical synthesis, or (iii) preparing cDNA sequences. DNA encoding p53 or RB fragments may be generated and used in any suitable way known to those of skill in the art, including by taking encoding DNA, identifying suitable restriction enzyme recognition sites either side of the portion to be expressed, and cutting out said portion from the DNA. The portion may then be operably linked to a suitable promoter in a standard commercially available expression system. Another recombinant approach is to amplify the relevant portion of the DNA with suitable PCR primers. Modifications to the p53 sequences can be made, e.g. using site directed mutagenesis, to lead to the expression of modified p53 peptide or to take account of codon preference in the host cells used to express the nucleic acid.

In order to obtain expression of the nucleic acid sequences, the sequences can be incorporated in a vector having one or more control sequences operably linked to the nucleic acid to control its expression. The vectors may include other sequences such as promoters or enhancers to drive the expression of the

inserted nucleic acid, nucleic acid sequences so that the polypeptide or peptide is produced as a fusion and/or nucleic acid encoding secretion signals so that the polypeptide produced in the host cell is secreted from the cell. Polypeptide can then be obtained by transforming the vectors into host cells in which the vector is functional, culturing the host cells so that the polypeptide is produced and recovering the polypeptide from the host cells or the surrounding medium. Prokaryotic and eukaryotic cells are used for this purpose in the art, including strains of E. coli, yeast, and eukaryotic cells such as COS or CHO cells.

Thus, the present invention also encompasses a method of making a polypeptide or peptide (as disclosed), the method including expression from nucleic acid encoding the polypeptide or peptide (generally nucleic acid according to the invention). This may conveniently be achieved by growing a host cell in culture, containing such a vector, under appropriate conditions which cause or allow expression of the polypeptide. Polypeptides and peptides may also be expressed in in vitro systems, such as reticulocyte lysate.

Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, eukaryotic cells such as mammalian and yeast, and baculovirus systems. Mammalian cell lines available in

the art for expression of a heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, COS cells and many others. A common, preferred bacterial host is E. coli.

Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. 'phage, or phagemid, as appropriate. For further details see, for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Current Protocols in Molecular Biology, Ausubel et al. eds., John Wiley & Sons, 1992.

Thus, a further aspect of the present invention provides a host cell containing heterologous nucleic acid as disclosed herein.

The nucleic acid of the invention may be integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques. The nucleic

acid may be on an extra-chromosomal vector within the cell, or otherwise identifiably heterologous or foreign to the cell.

A still further aspect provides a method which includes introducing the nucleic acid into a host cell. The introduction, which may (particularly for *in vitro* introduction) be generally referred to without limitation as "transformation", may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. vaccinia or, for insect cells, baculovirus. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage. As an alternative, direct injection of the nucleic acid could be employed.

Marker genes such as antibiotic resistance or sensitivity genes may be used in identifying clones containing nucleic acid of interest, as is well known in the art.

The introduction may be followed by causing or allowing expression from the nucleic acid, e.g. by culturing host cells (which may include cells actually transformed although more likely the cells will be descendants of the transformed cells) under conditions for expression of the gene, so that the encoded polypeptide (or peptide) is produced. If the

polypeptide is expressed coupled to an appropriate signal leader peptide it may be secreted from the cell into the culture medium. Following production by expression, a polypeptide or peptide may be isolated and/or purified from the host cell and/or culture medium, as the case may be, and subsequently used as desired, e.g. in the formulation of a composition which may include one or more additional components, such as a pharmaceutical composition which includes one or more pharmaceutically acceptable excipients, vehicles or carriers (e.g. see below).

Introduction of nucleic acid encoding a peptidyl molecule according to the present invention may take place in vivo by way of gene therapy, to disrupt or interfere with p53/RB interaction with a view to inducing apoptosis.

Thus, a host cell containing nucleic acid according to the present invention, e.g. as a result of introduction of the nucleic acid into the cell or into an ancestor of the cell and/or genetic alteration of the sequence endogenous to the cell or ancestor (which introduction or alteration may take place in vivo or ex vivo), may be comprised (e.g. in the soma) within an organism which is an animal, particularly a mammal, which may be human or non-human, such as rabbit, guinea pig, rat, mouse or other rodent, cat, dog, pig, sheep, goat, cattle or horse, or which is a bird, such as a chicken. Genetically modified or transgenic animals or

birds comprising such a cell are also provided as further aspects of the present invention.

This may have a therapeutic aim. (Gene therapy is discussed below. Also, the presence of a mutant, allele, derivative or variant sequence within cells of an organism, particularly when in place of a homologous endogenous sequence, may allow the organism to be used as a model in testing and/or studying substances which modulate activity of the encoded polypeptide in vitro or are otherwise indicated to be of therapeutic potential. Conveniently, however, assays for such substances may be carried out *in vitro*, within host cells or in cell-free systems.

Suitable screening methods are conventional in the art. They include techniques such as radioimmunosassay, scintillation proximetry assay and ELISA methods. Suitably either the RB protein or fragment or the p53 fragment or the analogue or variant thereof is immobilised whereupon the other is applied in the presence of the agents under test.

Scintillation Proximetry Assay in which a biotinylated RB protein fragment or the P53 fragment or variant is bound to streptavidin coated scintillant - impregnated beads (produced by Amersham). Binding of radiolabelled peptide is then measured by determination of radioactivity induced scintillation as the radioactive peptide binds to the immobilized fragment.

Agents which intercept this are thus inhibitors of RB/p53 interaction.

In one general aspect, the present invention provides an assay method for a substance with ability to disrupt interaction or binding between p53 and RB, the method including:

(a) bringing into contact a substance according to the invention including a peptide fragment of p53 or a variant or analogue thereof as disclosed, a substance including the relevant fragment of RB as disclosed or a variant or analogue thereof and a test compound, under conditions wherein, in the absence of the test compound being an inhibitor of interaction or binding of said substances, said substances interact or bind; and

(b) determining interaction or binding between said substances.

A test compound which disrupts, reduces, interferes with or wholly or partially abolishes binding or interaction between said substances (e.g. including a p53 fragment and including an RB fragment), and which may induce p53-mediated apoptosis, may thus be identified.

Another general aspect of the present invention provides an assay method for a substance able to bind the relevant region of RB or p53 as the case may be, the method including:

(a) bringing into contact a substance which includes

a peptide fragment of RB which interacts with p53 as disclosed, or a peptide fragment of p53 which interacts with RB as disclosed, or a variant or analogue thereof as disclosed, and a test compound; and

(b) determining binding between said substance and the test compound.

A test compound found to bind to the relevant portion of RB or p53, as the case may be, may be tested for ability to disrupt p53/RB interaction or binding and/or ability to induce p53-mediated apoptosis, or other property of p53 or activity of p53 or mediated by p53 as discussed already above.

An assay may include TBP, MDM2 or other molecule which binds p53 in a region to which RB binds, an assay method may include determining the effect of the test compound on binding between TBP, MDM2 or other molecule and p53. For certain embodiments it may be preferred to identify a compound able to inhibit RB binding to p53 without substantially affecting TBP, MDM2 or other molecule binding to p53, (i.e. a discriminatory compound), or without affecting mediation of a p53-mediated activity via such molecule.

A further assay according to the present invention tests for ability of a test compound to modulate promoter activity mediated by p53. p53 binding to a p53-binding sequence within a promoter can activate transcription from the promoter. The

oncogenic capacity of MDM2 protein is attributed to its ability to bind p53 and mask its transcriptional activation potential (see e.g. Martin *et al.*, *supra*, and references cited therein).

One such assay method includes:

(a) providing p53 or a fragment, variant or analogue thereof able to bind RB and able to activate transcription from a promoter including a p53 binding site, RB or a fragment, variant or analogue thereof able to bind p53, a test compound, and a reporter construct including a promoter which includes a p53 binding site and which is operably linked to a reporter sequence for transcription thereof, under conditions wherein, in the absence of the test compound being an inhibitor of RB/p53 binding, transcription of the reporter sequence is transcribed;

(b) determining promoter activity.

p53 activates transcription by binding a specific sequence within a promoter.

Wild-type p53 protein contains at least 4 functional domains, one of which (residues 102-290) binds to two copies of the consensus sequence 5'-PuPuPuC(A/T)(A/T)GpyPyPy-3'. The sequence GC3(GGACTTGCCT)2 may be used. A promoter including a p53 binding sequence, such as a consensus sequence is activated by wild-type p53, within the context of a

minimal promoter comprising a transcriptional start site and TATA Box. The p53 binding sequence may be included upstream of the minimal promoter or downstream.

By "promoter" is meant a sequence of nucleotides from which transcription may be initiated of DNA operably linked downstream (i.e. in the 3' direction on the sense strand of double-stranded DNA).

"Operably linked" means joined as part of the same nucleic acid molecule, suitably positioned and oriented for transcription to be initiated from the promoter. DNA operably linked to a promoter is "under transcriptional initiation regulation" of the promoter.

"Promoter activity" is used to refer to ability to initiate transcription. The level of promoter activity is quantifiable for instance by assessment of the amount of mRNA produced by transcription from the promoter or by assessment of the amount of protein product produced by translation of mRNA produced by transcription from the promoter. The amount of a specific mRNA present in an expression system may be determined for example using specific oligonucleotides which are able to hybridise with the mRNA and which are labelled or may be used in a specific amplification reaction such as the polymerase chain reaction. Use of a reporter gene as discussed further below facilitates determination of promoter activity by reference to protein production.

Generally, the gene may be transcribed into mRNA which may be translated into a peptide or polypeptide product which may be detected and preferably quantitated following expression. A gene whose encoded product may be assayed following expression may be termed a "reporter gene", i.e. a gene which "reports" on promoter activity.

A reporter gene preferably encodes an enzyme which catalyses a reaction which produces a detectable signal, preferably a visually detectable signal, such as a coloured product. Many examples are known, including β-galactosidase and luciferase. β-galactosidase activity may be assayed by production of blue colour on substrate, the assay being by eye or by use of a spectrophotometer to measure absorbance. Fluorescence, for example that produced as a result of luciferase activity, may be quantitated using a spectrophotometer. Radioactive assays may be used, for instance using chloramphenicol acetyltransferase, which may also be used in non-radioactive assays. The presence and/or amount of gene product resulting from expression from the reporter gene may be determined using a molecule able to bind the product, such as an antibody or fragment thereof. The binding molecule may be labelled directly or indirectly using any standard technique.

An assay method according to the present

invention may test for ability of p53 to induce G1 arrest and/or repair DNA. Cells may be UV-irradiated and p53-mediated induction of G1 arrest determined. DNA repair may be assayed *in vitro*.

Performance of an assay method according to the present invention may be followed by isolation and/or manufacture and/or use of a compound, substance or molecule which tests positive for ability to interfere with the p53/RB interaction or binding and/or induce p53-mediated apoptosis or other p53-mediated activity or activity or p53 or property of p53.

Ability of a compound to enhance the ability of p53 to mediate apoptosis in the presence of RB may be determined using an assay as disclosed in Haupt *et al.* (*Oncogene* (1995) 10: 1563-1571). p53 and RB may be expressed from appropriate encoding nucleic acid within host cells such as HeLa cells. Haupt *et al.* report that overexpression of p53 induces apoptotic cell death, while co-expression of RB results in significant protection of cells from p53-mediated apoptosis. A substance able to interfere with the interaction between p53 and RB established for the first time herein may be useful in inducing p53-mediated apoptosis.

The precise format of an assay of the invention may be varied by those of skill in the art using

routine skill and knowledge. For example, interaction between substances may be studied *in vitro* by labelling one with a detectable label and bringing it into contact with the other which has been immobilised on a solid support. Suitable detectable labels, especially for petidyl substances include ^{35}S-methionine which may be incorporated into recombinantly produced peptides and polypeptides. Recombinantly produced peptides and polypeptides may also be expressed as a fusion protein containing an epitope which can be labelled with an antibody.

The protein which is immobilized on a solid support may be immobilized using an antibody against that protein bound to a solid support or via other technologies which are known *per se*. A preferred *in vitro* interaction may utilise a fusion protein including glutathione-S-transferase (GST). This may be immobilized on glutathione agarose beads. In an *in vitro* assay format of the type described above a test compound can be assayed by determining its ability to diminish the amount of labelled peptide or polypeptide which binds to the immobilized GST-fusion polypeptide. This may be determined by fractionating the glutathione-agarose beads by SDS-polyacrylamide gel electrophoresis. Alternatively, the beads may be rinsed to remove unbound protein and the amount of protein which has bound can be determined by counting the amount of label present in, for example, a suitable

scintillation counter.

An assay according to the present invention may also take the form of an *in vivo* assay. The *in vivo* assay may be performed in a cell line such as a yeast strain or mammalian cell line in which the relevant polypeptides or peptides are expressed from one or more vectors introduced into the cell.

The ability of a test compound to interact with or bind the relevant portion of p53 or RB, or to disrupt interaction or binding between p53 or RB, may be determined using a so-called two-hybid assay.

For example, a polypeptide or peptide containing a fragment or p53 or RB as the case may be, or a peptidyl analogue or variant thereof as disclosed, may be fused to a DNA binding domain such as that of the yeast transcription factor GAL 4. The GAL 4 transcription factor includes two functional domains. These domains are the DNA binding domain (GAL4DBD) and the GAL4 transcriptional activation domain (GAL4TAD). By fusing one polypeptide or peptide to one of those domains and another polypeptide or peptide to the respective counterpart, a functional GAL 4 transcription factor is restored only when two polypeptides or peptides of interest interact. Thus, interaction of the polypeptides or peptides may be measured by the use of a reporter gene probably linked to a GAL 4 DNA binding site which is capable of

activating transcription of said reporter gene. This assay format is described by Fields and Song, 1989, Nature 340; 245-246. This type of assay format can be used in both mammalian cells and in yeast. Other combinations of DNA binding domain and transcriptional activation domain are available in the art and may be preferred, such as the LexA DNA binding domain and the VP60 transcriptional activation domain.

To take a Lex/VP60 two hybrid screen by way of example for the purpose of illustration, yeast or mammalian cells may be transformed with a reporter gene construction which expresses a selective marker protein (e.g. encoding β-galactosidase or luciferase). The promoter of that gene is designed such that it contains binding site for the LexA DNA-binding protein. Gene expression from that plasmid is usually very low. Two more expression vectors may be transformed into the yeast containing the selectable marker expression plasmid, one containing the coding sequence for the full length LexA gene linked to a multiple cloning site. This multiple cloning site is used to clone a gene of interest, i.e. encoding a p53 or RB polypeptide or peptide in accordance with the present invention, in frame on to the LexA coding region. The second expression vector then contains the activation domain of the herpes simplex transactivator VP16 fused to a test peptide sequence or more preferably a library of sequences encoding peptides with diverse e.g. random

sequences. Those two plasmids facilitate expression from the reporter construct containing the selectable marker only when the LexA fusion construct interacts with a polypeptide or peptide sequence derived from the peptide library.

A modification of this when looking for peptides which interfere with interaction between a p53 polypeptide or peptide and an RB polypeptide or peptide, employs the p53 or RB polypeptide or peptide as a fusion with the LexA DNA binding domain, and the counterpart RB or p53 polypeptide or peptide as a fusion with VP60, and involves a third expression cassette, which may be on a separate expression vector, from which a peptide or a library of peptides of diverse and/or random sequence may be expressed. A reduction in reporter gene expression (e.g. in the case of β-galactosidase a weakening of the blue colour) results from the presence of a peptide which disrupts the p53/RB interaction, which interaction is required for transcriptional activation of the β-galactosidase gene.

As noted, instead of using LexA and VP60, other similar combinations of proteins which together form a functional transcriptional activator may be used, such as the GAL4 DNA binding domain and the GAL4 transcriptional activation domain.

When performing a two hybrid assay to look for substances which interfere with the interaction between

two polypeptides or peptides it may be preferred to use mammalian cells instead of yeast cells. The same principles apply and appropriate methods are well known to those skilled in the art.

The amount of test substance or compound which may be added to an assay of the invention will normally be determined by trial and error depending upon the type of compound used. Typically, from about 0.01 to 100 nM concentrations of putative inhibitor compound may be used, for example from 0.1 to 10 nM. Greater concentrations may be used when a peptide is the test substance.

Compounds which may be used may be natural or synthetic chemical compounds used in drug screening programmes. Extracts of plants which contain several characterised or uncharacterised components may also be used. As noted, a further class of putative inhibitor compounds can be derived from the p53 or RB polypeptide.

Antibodies directed to the site of interaction in either protein form a further class of putative inhibitor compounds. Candidate inhibitor antibodies may be characterised and their binding regions determined to provide single chain antibodies and fragments thereof which are responsible for disrupting the interaction.

Antibodies may be obtained using techniques which are standard in the art. Methods of producing antibodies include immunising a mammal (e.g. mouse, rat, rabbit, horse, goat, sheep or monkey) with the protein or a fragment thereof. Antibodies may be obtained from immunised animals using any of a variety of techniques known in the art, and screened, preferably using binding of antibody to antigen of interest. For instance, Western blotting techniques or immunoprecipitation may be used (Armitage *et al.*, 1992, Nature 357: 80-82). Isolation of antibodies and/or antibody-producing cells from an animal may be accompanied by a step of sacrificing the animal.

As an alternative or supplement to immunising a mammal with a peptide, an antibody specific for a protein may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, e.g. using lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047. The library may be naive, that is constructed from sequences obtained from an organism which has not been immunised with any of the proteins (or fragments), or may be one constructed using sequences obtained from an organism which has been exposed to the antigen of interest.

Antibodies according to the present invention may be modified in a number of ways. Indeed the term

"antibody" should be construed as covering any binding substance having a binding domain with the required specificity. Thus the invention covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including synthetic molecules and molecules whose shape mimicks that of an antibody enabling it to bind an antigen or epitope.

Example antibody fragments, capable of binding an antigen or other binding partner are the Fab fragment consisting of the VL, VH, Cl and CH1 domains; the Fd fragment consisting of the VH and CH1 domains; the Fv fragment consisting of the VL and VH domains of a single arm of an antibody; the dAb fragment which consists of a VH domain; isolated CDR regions and F(ab')2 fragments, a bivalent fragment including two Fab fragments linked by a disulphide bridge at the hinge region. Single chain Fv fragments are also included.

A hybridoma producing a monoclonal antibody according to the present invention may be subject to genetic mutation or other changes. It will further be understood by those skilled in the art that a monoclonal antibody can be subjected to the techniques of recombinant DNA technology to produce other antibodies or chimeric molecules which retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementarity determining

regions (CDRs), of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP184187A, GB 2188638A or EP-A-0239400. Cloning and expression of chimeric antibodies are described in EP-A-0120694 and EP-A-0125023.

Hybridomas capable of producing antibody with desired binding characteristics are within the scope of the present invention, as are host cells, eukaryotic or prokaryotic, containing nucleic acid encoding antibodies (including antibody fragments) and capable of their expression. The invention also provides methods of production of the antibodies including growing a cell capable of producing the antibody under conditions in which the antibody is produced, and preferably secreted.

The reactivities of antibodies on a sample may be determined by any appropriate means. Tagging with individual reporter molecules is one possibility. The reporter molecules may directly or indirectly generate detectable, and preferably measurable, signals. The linkage of reporter molecules may be directly or indirectly, covalently, e.g. via a peptide bond or non-covalently. Linkage via a peptide bond may be as a result of recombinant expression of a gene fusion encoding antibody and reporter molecule.

One favoured mode is by covalent linkage of each antibody with an individual fluorochrome, phosphor or

laser dye with spectrally isolated absorption or emission characteristics. Suitable fluorochromes include fluorescein, rhodamine, phycoerythrin and Texas Red. Suitable chromogenic dyes include diaminobenzidine.

Other reporters include macromolecular colloidal particles or particulate material such as latex beads that are coloured, magnetic or paramagnetic, and biologically or chemically active agents that can directly or indirectly cause detectable signals to be visually observed, electronically detected or otherwise recorded. These molecules may be enzymes which catalyse reactions that develop or change colours or cause changes in electrical properties, for example. They may be molecularly excitable, such that electronic transitions between energy states result in characteristic spectral absorptions or emissions. They may include chemical entities used in conjunction with biosensors. Biotin/avidin or biotin/streptavidin and alkaline phosphatase detection systems may be employed.

The mode of determining binding is not a feature of the present invention and those skilled in the art are able to choose a suitable mode according to their preference and general knowledge.

Antibodies may also be used in purifying and/or isolating a polypeptide or peptide according to the present invention, for instance following production of

the polypeptide or peptide by expression from encoding nucleic acid therefor. Antibodies may be useful in a therapeutic context (which may include prophylaxis) to disrupt p53/RB interaction with a view to inducing p53-mediated apoptosis. Antibodies can for instance be micro-injected into cells, e.g. at a tumour site.

Other candidate inhibitor compounds may be based on modelling the 3-dimensional structure of a polypeptide or peptide fragment and using rational drug design to provide potential inhibitor compounds with particular molecular shape, size and charge characteristics.

A compound found to have the ability to modulate p53-mediate apoptosis has therapeutic potential in anti-tumour treatment, and may be used in combination with any other anti-tumour compound. In such a case, the assay of the invention, when conducted *in vivo*, need not measure the degree of inhibition of binding or of induction of apoptosis caused by the compound being tested. Instead the effect on tumorigenicity and/or cell viability may be measured. It may be that such a modified assay is run in parallel with or subsequent to the main assay of the invention in order to confirm that any effect on tumorigenicity or and/or cell viability is as a result of the inhibition of binding or interaction between p53 and RB caused by said

inhibitor compound and not merely a general toxic effect.

Following identification of a substance or agent which modulates or affects p53 activity, the substance or agent may be investigated further. Furthermore, it may be manufactured and/or used in preparation, i.e. manufacture or formulation, of a composition such as a medicament, pharmaceutical composition or drug. These may be administered to individuals.

As noted, the agent may be peptidyl, e.g. a peptide which includes a sequence as recited above, or may be a functional analogue of such a peptide.

As used herein, the expression "functional analogue" relates to peptide variants or organic compounds having the same functional activity as the peptide in question, in particular which interfere with the binding between p53 and RB. Examples of such analogues include chemical compounds which are modelled to resemble the three dimensional structure of the p53 or RB domain in the contact area, and in particular the arrangement of the key amino acid residues identified above as they appear in human p53.

Suitable modelling techniques are known in the art. This includes the design of so-called "mimetics" which involves the study of the functional interactions fluorogenic oligonucleotide the molecules and the

design of compounds which contain functional groups arranged in such a manner that they could reproduced those interactions.

The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a particular method of administration, e.g. peptides are not well suited as active agents for oral compositions as they tend to be quickly degraded by proteases in the alimentary canal. Mimetic design, synthesis and testing may be used to avoid randomly screening large number of molecules for a target property.

There are several steps commonly taken in the design of a mimetic from a compound having a given target property. Firstly, the particular parts of the compound that are critical and/or important in determining the target property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, e.g. by substituting each residue in turn. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is modelled to according its physical properties, e.g. stereochemistry, bonding, size and/or

charge, using data from a range of sources, e.g. spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

In a variant of this approach, the three-dimensional structure of the ligand and its binding partner are modelled. This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to take account of this the design of the mimetic.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological activity of the lead compound. The mimetic or mimetics found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing.

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Further optimisation or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing.

Mimetics of this type together with their use in therapy form a further aspect of the invention.

Generally, an inhibitor according to the present invention is provided in an isolated and/or purified form, i.e. substantially pure. This may include being in a composition where it represents at least about 90% active ingredient, more preferably at least about 95%, more preferably at least about 98%. Such a composition may, however, include inert carrier materials or other pharmaceutically and physiologicaly acceptable excipients. As noted below, a composition according to the present invention may include in addition to an inhibitor compound as disclosed, one or more other molecules of therapeutic use, such as an anti-tumour agent.

The present invention extends in various aspects not only to a substance identified as a modulator of p53/RB interaction and/or p53-mediated apoptosis or other p53-mediated activity, property or pathway in accordance with what is disclosed herein, but also a pharmaceutical composition, medicament, drug or other composition comprising such a substance, a method comprising administration of such a composition to a

patient, e.g. for anti-tumour or other anti-proliferative treatment, which may include preventative treatment, use of such a substance in manufacture of a composition for administration, e.g. for anti-tumour or other anti-proliferative treatment, and a method of making a pharmaceutical composition comprising admixing such a substance with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients.

A aubstance according to the present invention such as an inhibitor of p53/RB interaction or binding may be provided for use in a method of treatment of the human or animal body by therapy which induces p53-mediated apoptosis or other p53-mediated activity in cells, e.g. tumour cells.

Thus the invention further provides a method of inducing apoptosis or other p53-mediated activity in a cell, or affecting a property of p53, which includes administering an agent which inhibits or blocks the binding of p53 to RB protein, such a method being useful in treatment of cancer or other diseases or disorders including malignancies where cell-apoptosis is desirable.

The invention further provides a method of treating tumours which includes administering to a patient an agent which interferes with the binding p53

to RB protein.

Whether it is a polypeptide, antibody, peptide, nucleic acid molecule, small molecule, mimetic or other pharmaceutically useful compound according to the present invention that is to be given to an individual, administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practioners and other medical doctors.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may include, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the

efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. cutaneous, subcutaneous or intravenous.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Examples of techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed), 1980.

The agent may be administered in a localised manner to a tumour site or other desired site or may be delivered in a manner in which it targets tumour or other cells.

Targeting therapies may be used to deliver the active agent more specifically to certain types of cell, by the use of targeting systems such as antibody or cell specific ligands. Targeting may be desirable for a variety of reasons, for example if the agent is unacceptably toxic, or if it would otherwise require too high a dosage, or if it would not otherwise be able to enter the target cells.

Instead of administering these agents directly, they may be produced in the target cells by expression from an encoding gene introduced into the cells, eg in a viral vector (a variant of the VDEPT technique - see below). The vector may targeted to the specific cells to be treated, or it may contain regulatory elements which are switched on more or less selectively by the target cells.

The agent may be administered in a precursor form, for conversion to the active form by an activating agent produced in, or targeted to, the cells to be treated. This type of approach is sometimes known as ADEPT or VDEPT, the former involving targeting the activating agent to the cells by conjugation to a cell-specific antibody, while the latter involves producing the activating agent, e.g. an enzyme, in a

vector by expression from encoding DNA in a viral vector (see for example, EP-A-415731 and WO 90/07936).

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated, such as cancer, virus infection or any other condition in which p53-mediated apoptosis is desirable.

Nucleic acid according to the present invention, encoding a polypeptide or peptide able to interfere with p53/RB interaction or binding and/or induce or modulate p53-mediated apoptosis or other p53-mediated cellular pathway or function, and/or affect a property of p53 such as its stability/half-life, may be used in methods of gene therapy, for instance in treatment of individuals with the aim of preventing or curing (wholly or partially) a tumour e.g. in cancer, or other disorder involving loss of proper regulation of the cell-cycle and/or cell growth, or other disorder in which specific cell death is desirable, such as in certain viral infections.

Vectors such as viral vectors have been used in the prior art to introduce nucleic acid into a wide variety of different target cells. Typically the vectors are exposed to the target cells so that transfection can take place in a sufficient proportion of the cells to provide a useful therapeutic or

prophylactic effect from the expression of the desired polypeptide. The transfected nucleic acid may be permanently incorporated into the genome of each of the targeted tumour cells, providing long lasting effect, or alternatively the treatment may have to be repeated periodically.

A variety of vectors, both viral vectors and plasmid vectors, are known in the art, see US Patent No. 5,252,479 and WO 93/07282. In particular, a number of viruses have been used as gene transfer vectors, including papovaviruses, such as SV40, vaccinia virus, herpesviruses, including HSV and EBV, and retroviruses. Many gene therapy protocols in the prior art have used disabled murine retroviruses.

As an alternative to the use of viral vectors other known methods of introducing nucleic acid into cells includes electroporation, calcium phosphate co-precipitation, mechanical techniques such as microinjection, transfer mediated by liposomes and direct DNA uptake and receptor-mediated DNA transfer.

Receptor-mediated gene transfer, in which the nucleic acid is linked to a protein ligand via polylysine, with the ligand being specific for a receptor present on the surface of the target cells, is an example of a technique for specifically targeting nucleic acid to particular cells.

A polypeptide, peptide or other substance able to

interfere with the interaction of the relevant polypeptide, peptide or other substance as disclosed herein, or a nucleic acid molecule encoding a peptidyl such molecule, may be provided in a kit, e.g. sealed in a suitable container which protects its contents from the external environment. Such a kit may include instructions for use.

Various aspects and embodiments of the present invention will now be illustrated by way of example with reference to the accompanying diagrammatic drawings in which:

Figure 1 shows results of immunoprecipitation assays indicating that RB and p53 interact *in vivo*.

Figure 2 shows the results of GST-pull-down experiments in which binding of various portions of p53 with a portion of RB (residues 379-928) was investigated. The % of input RB portion bound is indicated for each p53 fragment. The major binding site of RB on p53 is at the N-terminus of p53 but there is also affinity to the C-terminus.

Figure 3 shows sequence information on regions of p53 which interact with RB:

Figure 3A shows that the mouse p53 activation domain shows homology with the E2F protein activation domain, by alignment of amino acids 380-437 of E2F1 with the mouse p53 activation domain residues 11-71.

Figure 3B shows that the human p53 activation domain and part of the C-terminal domain similarly show homology to the E2F protein activation domain, by alignment of amino acids 373-437 of the E2F protein with p53 activation domain (amino acids 1-71) and part of the p53 C-terminal domain (amino acids 370-393). Residues in E2F involved in RB binding are indicated, as well as residues conserved between the E2F and p53 domains. Homology to the RB binding site is indicated. The point and deletion mutants generated in the p53 activation domain and used in various experimental examples described herein are indicated underneath.

Figure 3C shows the amino acid sequence and encoding nucleic acid sequenc of human p53. In addition to the RB binding sites within the N-terminal region, amino acid residues 290-393 of the C-terminal region include a RB binding site.

Figure 4 shows as a percentage relative to wild-type, the binding of variously mutated forms of p53ΔC (residues 1-344) to a portion of RB (residues 379-928), as ascertained in GST-pull-down experiments. Mutants which substantially affect RB binding are the Δ40-45 and also 14/19 and 22/23 (see Figure 3 for details of the mutations.

Figure 5 shows results of experiments which indicate that p53 mutants which are defective in RB binding are also defective for p53-induced transcriptional repression. This activity has been

correlated with the ability of p53 to induce apoptosis.

Figure 6 shows results of experiments in which a cellular phenotype indicative of apoptosis was determined for variously mutated forms of the N-terminal fragment of p53. The 40-45 deletion is defective for the induction of apoptosis by p53, consistent with binding of RB to p53 resulting in protection of cells from apoptosis.

Figure 7 shows the results of pull-down experiments using GST-TBP and translated p53, lanes 1 to 6: p53 WT, p53 del C (amino acids 1 to 290); p53 14-19, p53 del30-34, p53 del 40-45, p53 del 67-72. The results show reduced TBP binding to p53 del 40-45 (lane 5) compared with the others.

Figure 8 illustrates diagrammatically an overview of interference with the RB-p53 mechanism in accordance with the present invention leading to apoptosis.

Various other aspects and embodiments will be apparent to those skilled in the art. All documents mentioned herein are incorporated by reference.

As illustrated hereinafter, the inventor has discovered and characterised binding regions responsible for interaction between p53 and RB proteins.

EXAMPLE 1

Binding of a region of RB residues 379-928 to A region of p53 residues 1-73 was identified using a GST pull-down assay as follows.

Five hundred nanograms of a GST-RB 379-928 fusion protein were linked to glutathione agarose beads, preincubated with bovine serum albumin (final concentration, 1mg/ml) at room temperature (RT) for 5 min and then rocked for 1 h at RT with 2μl of *in vitro* translated and radiolabelled p53 protein in 200μl of Z' buffer (25mM Hepes, pH 7.5; 12.5mM $MgCl_2$; 20% glycerol; 0/1% NP-40; 150 mM KCl; 20 μM $ZnSO_4$). The beads were then washed three times in 1.5ml of NETN buffer (150 mM NaCl; 1mM EDTA; 0.5% NP-40; 20 mM TRis-HCL, pH8.0), pelleted at 500 x g for 30 s and boiled in SDS-PAGE sample buffer. Bound proteins were resolved by SDS-PAGE and subjected to autoradiography.

This procedure showed that a portion of the p53 protein binds to GST-RB but not to a control GST protein.

Binding of GST-p53 1-73 to *in vitro* translated RB-379-928 has also been demonstrated in a GST pull-down assay using the above method.

EXAMPLE 2

As shown in Figure 1, RB and p53 co-immunoprecipitate.

U2OS cells were transfected with 6 μg of CMV-p53

and 6 μg of CMV-RB expression vectors. Whole cells extracts were then immunoprecipitated using a p53 or Rb antibodies or an irrelevant Ab (HA) as indicated. Immunoprecipitates were assayed by western blotting for the presence of RB as indicated by arrows.

Antibody to p53 co-precipitates RB, indicating the two proteins interact *in vivo*.

EXAMPLE 3

As shown by the results illustrated in Table 1, RB and p53 interact in a yeast two hybrid system, involving the standard lexA and VP16 proteins.

The DY 1641 reporter strain was transformed by the indicated plasmids.

To assay β-galactosidase activity, individual transformants were grown on minimal medium without histidine, tryptophane and leucine to select for growth. The colonies were lifted on nitrocellulose filters and placed on a 3MM filter paper soaked in Z buffer containing 0.5 mg/ml of X-gal. The filters were incubated at 30°C for 1 hour.

A positive blue colour was obtained with when the lexA and VP16 proteins constituted a functional transcriptional activator which initiated β-galactosidase expression as a result of interaction between the RB and p53.

EXAMPLE 4

Figure 2 illustrates the results of experiments investigating P53 domains required for RB binding.

P53 polypeptides with N- and C-terminal deletions were translated *in vitro* and labelled with [^{35} S] methionine. Each of these proteins was incubated with GST-RB (379-928) linked to glutathione-agarose beads. After centrifugation of the beads the p53 protein coprecipitated with GST-RB was resolved on a 12%/SDS polyacrylamide gel and quantified by phosphorimager.

Strong interaction is shown for p53 fragments including the N-terminus, while weaker interaction is evidenced for the C-terminus.

EXAMPLE 5

Figure 4 illustrates the results of further experiments investigating binding of p53 N-terminal sequences with RB.

Pull-downs were performed as in Example 4, except that the p53 translated proteins are missing the C-terminal domain (amino-acids 1-344).

Results are shown as percentages of the interaction with the wild-type sequence, for variously mutated fragments.

From the alignment of p53 (1-71) and E2F (373-437) (Figure 3A and Figure 3B) a binding site for RB in p53 is predicted to be within p53 residues 43-60 for

the mouse and 40-50 for the human. A deletion of sequences in this region of human p53 (Δ40-45 Figure 3) disrupt the ability of RB to bind p53 (Figure 4). In addition, there are two other mutations in the human (14/19 and 22/23 (Figure 3) which also disrupt RB binding (Figure 4). A distinct binding site for RB is also present at the C-terminus of p53, within residues 290-393 (Figure 2). In this region there is a sequence which shows similarity to the RB binding site in E2F (Figure 3B, alignment of p53 370-393).

EXAMPLE 6

Transcriptional repression by wild type and mutant p53 proteins was investigated.

Saos2 cells were transfected with 1 μg of pSV2-CAT reporter and 5 μg of CMV-p53 expression vector, with various mutant p53 fragments as indicated. Following a CAT assay results were quantified using a phosphorimager.

The activity of the reporter in absence of p53 was normalized to 100%, and the p53 repression expressed in % (top panel). An average of three experiments is shown in Figure 5.

The level of p53 expression was assayed by western blotting with the p421 antibody (lower panel).

It can be seen that the lower band in the lane for the 22/23 mutant (within the MDM2 binding site) is darker, indicative of increased p53 protein stability.

EXAMPLE 7

Cells expressing different mutant p53 fragments were assayed for evidence of apoptosis.

72 hours after transfection of the Saos2 cells wild type or mutants (as indicated), the level of apoptosis in the p53 transfected cells was measured as accumulation of cells with a sub-G1 DNA content.

The graph in Figure 6 shows the percentage of p53 expressing cells in sub G1 apoptotic population.

Evidence is provided for RB binding being linked to protecting cells from p53-induced apoptosis. Mutagenesis of the RB binding residues affects the ability of p53 to induce apoptosis (Figure 6) and its ability to repress transcription (Figure 5), a function which correlates with induction of apoptosis.

TABLE 1

plasmids	growth on histidine minus medium	color
plexA-RB (379-928) +pVP16	-	white
plexA-RB (379-928) + pVP16-p53	+	blue
plexA-RB (379-928)	-	white

CLAIMS:

1. An assay method for identifying a compound with ability to modulate interaction or binding between p53 and Retinoblastoma protein (RB), the method including:

(a) bringing into contact p53 or a fragment thereof, RB or a fragment thereof, and a test compound, under conditions wherein, in the absence of the test compound being an inhibitor of interaction or binding of p53 and RB, the p53 or fragment thereof and the RB or fragment thereof interact or bind; and

(b) determining interaction or binding between the p53 or fragment thereof and the RB or fragment thereof.

2. An assay method for identifying a compound with ability to modulate interaction or binding between p53 and Retinoblastoma protein (RB), the method including:

(a) bringing into contact a substance which includes p53 or a fragment thereof from within amino acids 1-73 or a fragment thereof from within amino acids 290-293, or a variant or analogue of fragment, a substance including amino acids 369-928 of RB, or a variant or analogue thereof, and a test compound, under conditions wherein, in the absence of the test compound being an inhibitor of interaction or binding of said substances, said substances interact or bind; and

(b) determining interaction or binding between said substances.

3. An assay method according to claim 1 or claim 2 wherein the fragment, variant or analogue of p53 includes the amino acid sequence

- D L - L - - D - - E

4. An assay method according to claim 3 wherein the fragment, variant or analogue of p53 includes the amino acid sequence

M D D L M L S P D D I E

5. An assay method according to claim 1 or claim 2 wherein the fragment, variant or analogue of p53 includes the amino acid sequence

- D L - L - - D - - E - F - - - - E.

6. An assay method according to claim 5 wherein the fragment, variant or analogue of p53 includes the amino acid sequence

D D L L L P Q D V E E F F E G P S E.

7. An assay method according to claim 1 or claim 2 wherein the fragment, variant or analogue of p53 includes the amino acid sequence

K S K K G Q S T S R H K K L M F K T E G P D S D.

8. An assay method for identifying a compound able to bind a region within amino acids 369-928 of Retinoblastoma protein (RB) which binds p53, the method

including:

(a) bringing into contact a substance which includes amino acids 369-928 of RB or a fragment, variant or analogue thereof able to interact with or bind p53, and a test compound; and

(b) determining binding between said substance and the test compound.

9. A method according to claim 8 wherein a compound found to be able to bind said region of RB is tested for ability to modulate interaction or binding between RB and p53 or a fragment, variant or analogue thereof able to bind RB.

10. A method according to any of claims 1 to 9 wherein a compound is additionally tested or ability to modulate a p53-mediated activity or property of p53.

11. A method according to claim 10 wherein the p53-mediated activity is transcriptional activation from a promoter including a p53 binding site.

12. A method according to claim 10 wherein the p53-mediated activity includes induction of apoptosis.

13. A method of identifying a compound able to modulate a p53-mediated activity of p53 by interfering with interaction between p53 and Retinoblastoma protein

(RB), the method including:

(a) contacting cells with a test compound;

(b) determining modulation of the p53-mediated activity in the cells;

(c) selecting a compound able to modulate the p53-mediated activity as determined in step (b);

(d) determining ability of the compound selected in step (c) to interfere with interaction between p53 and RB.

14. A method according to claim 13 wherein the p53-mediated activity includes induction of apoptosis.

15. A method which includes, following identification of a compound as being able to interfere with interaction or binding between p53 and Retinoblastoma protein (RB) and/or induce apoptosis in accordance with any of claims 1 to 14, formulation of the compound into a composition including at least one additional component.

16. Use of p53 or a fragment thereof from within amino acids 1-73 or a fragment thereof from within amino acids 290-293, or a variant or analogue of a said fragment able to interact with or bind Retinoblastoma protein (RB), in screening for compounds able to modulate interaction or binding between p53 and RB.

17. Use of p53 or a fragment thereof from within amino acids 1-73 or a fragment thereof from within amino acids 290-293, or a variant or analogue of a said fragment able to interact with or bind Retinoblastoma protein (RB), in screening for compounds able to modulate a p53-mediated activity or property of p53.

18. Use according to claim 17 wherein the p53-mediated activity includes induction of apoptosis.

19. Use according to claim 16 or claim 17 wherein the fragment, variant or analogue of p53 includes the amino acid sequence

- D L - L - - D - - E.

20. Use according to claim 19 wherein the fragment, variant or analogue of p53 includes the amino acid sequence

M D D L M L S P D D I E.

21. Use according to claim 16 or claim 17 wherein the fragment, variant or analogue of p53 includes the amino acid sequence

- D L - L - - D - - E - F - - - - E.

22. Use according to claim 21 wherein the fragment, variant or analogue of p53 includes the amino acid sequence

D D L L L P Q D V E E F F E G P S E.

23. Use according to claim 16 or claim 17 wherein the fragment, variant or analogue of p53 includes the amino acid sequence

K S K K G Q S T S R H K K L M F K T E G P D S D.

24. An inhibitor of interaction between p53 and Retinoblastoma protein (RB), which inhibitor is:

(i) a peptide which is a fragment of p53 from within amino acids 1-73;

(ii) a peptide which is a fragment of p53 from within amino acids 290-393;

(iii) a peptide or about 40 amino acids or less which is a sequence variant of said amino acid sequence of peptide (i) or peptide (ii), by way of addition, substitution, insertion and/or deletion of one or more amino acids; or

(iv) a non-peptidyl mimetic of all or part of said amino acid sequence of peptide (i) or peptide (ii).

25. An inhibitor according to claim 24 which includes the amino acid sequence

- D L - L - - D - - E

26. An inhibitor according to claim 25 which includes the amino acid sequence

M D D L M L S P D D I E

27. An inhibitor according to claim 25 which includes the amino acid sequence

- D L - L - - D - - E - F - - - - E.

28. An inhibitor according to claim 27 which includes the amino acid sequence

D D L L L P Q D V E E F F E G P S E.

29. An inhibitor according to claim 24 consisting essentially of a peptide with the amino acid sequence

- - D L - L - - D - - E

or a fragment thereof.

30. An inhibitor according to claim 29 which consists essentially of a peptide with the amino acid sequence

M D D L M L S P D D I E

or a fragment thereof.

31. An inhibitor according to claim 24 consisting essentially of a peptide with the amino acid sequence

- D L - L - - D - - E - F - - - - E

or a fragment thereof.

32. An inhibitor according to claim 31 consisting essentially of a peptide with the amino acid sequence

D D L L L P Q D V E E F F E G P S E

or a fragment thereof.

33. An inhibitor according to claim 24 which includes the amino acid sequence

K S K K G Q S T S R H K K L M F K T E G P D S D.

34. An inhibitor according to claim 24 consisting essentially of a pepide with the amino acid sequence

K S K K G Q S T S R H K K L M F K T E G P D S D

or a fragment thereof.

35. An inhibitor according to claim 24 which includes the amino acid sequence

E P P L S Q E T F S D L W K L I P E N.

36. An inhibitor according to claim 24 which consists essentially of the amino acid sequence

E P P L S Q E T F S D L W K L I P E N

or a fragment thereof.

37. A polypeptide or peptide including a peptidyl inhibitor according to any of claims 24 to 36 and an additional amino acid sequence heterologous to p53.

38. A composition including an inhibitor or polypeptide or peptide according to any of claims 24 to 37 and at least one additional component.

39. A composition according to claim 38 wherein the inhibitor is coupled to a carrier for delivery to

cells.

40. A composition according to claim 38 or claim 39 including a physiologically acceptable carrier or excipient.

41. Use of an inhibitor of interaction between p53 and Retinoblastoma protein (RB) in the manufacture of a medicament for modulating a p53-mediated activity or property of p53.

42. Use according to claim 41 wherein the p53-mediated activity includes induction of apoptosis.

43. Use of an inhibitor according to any of claims 24 to 36 in the manufacture of a medicament for interfering with interaction between p53 and Retinoblastoma protein (RB).

44. Use of an inhibitor according to any of claims 24 to 36 in the manufacture of a medicament for modulating a p53-mediated activity or property of p53.

45. Use according to claim 44 wherein the p53-mediated activity includes induction of apoptosis.

46. An inhibitor of interaction between p53 and Retinoblastoma protein (RB) for use in a method for

treatment of the human or animal body by therapy which modulates a p53-mediated activity or property of p53.

47. An inhibitor according to claim 46 wherein the p53-mediated activity includes induction of apoptosis.

48. An inhibitor according to claim 47 wherein said apoptosis is induced in tumour cells.

49. An inhibitor according to any of claims 24 to 36 for use in a method for treatment of the human or animal body by therapy which interferes with interaction between p53 and Retinoblastoma protein (RB).

50. An inhibitor according to any of claims 24 to 36 for use in a method of treatment of the human or animal body by therapy which modulates a p53-mediated activity or property of p53.

51. An inhibitor according to claim 50 wherein the p53-mediated activity includes induction of apoptosis.

52. An inhibitor according to claim 51 wherein said apoptosis is induced in tumour cells.

53. A method of modulating a p53-mediated activity or property of p53, the method including contacting p53

and/or Retinoblastoma protein (RB) with an inhibitor of interaction between p53 and RB.

54. A method according to claim 53 wherein the p53-mediated activity includes induction of apoptosis.

55. A method of interfering with interaction between p53 and Retinoblastoma protein (RB), the method including contacting p53 and/or RB with an inhibitor according to any of claims 24 to 36, a polypeptide or peptide according to claim 37, or a composition according to any of claims 38 to 40.

56. A method of modulating p53-mediated activity or property of p53, the method including contacting p53 and/or Retinoblastoma protein (RB) with an inhibitor according to any of claims 24 to 36, a polypeptide or peptide according to claim 37, or a composition according to any of claims 38 to 40.

57. A method according to claim 56 wherein the p53-mediated activity includes induction of apoptosis.

58. A method according to any of claims 53 to 57 which takes place *in vitro* or *ex vivo*.

59. A method according to any of claims 53 to 57 which takes place *in vivo*.

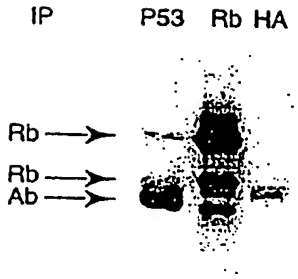


Figure 1

Figure 2

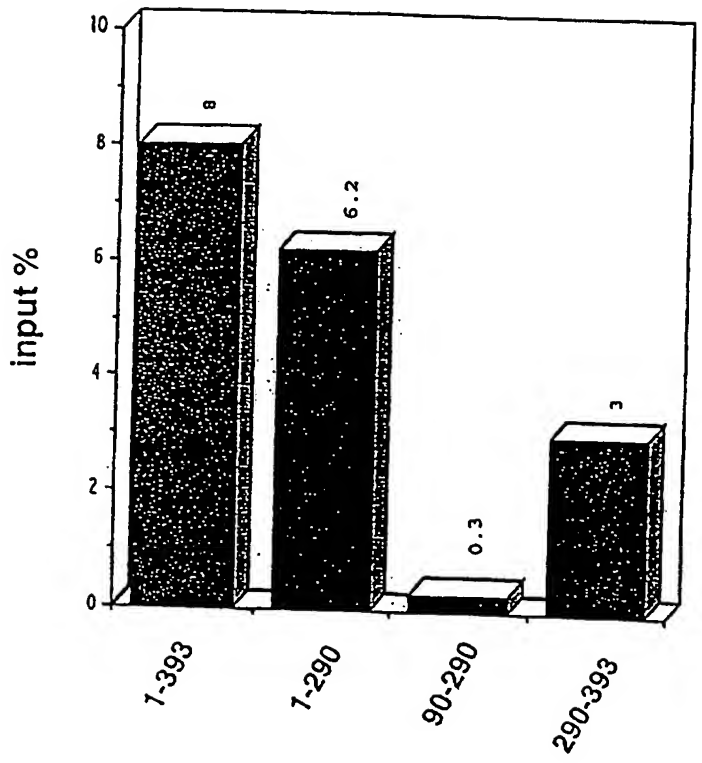


Figure 3A

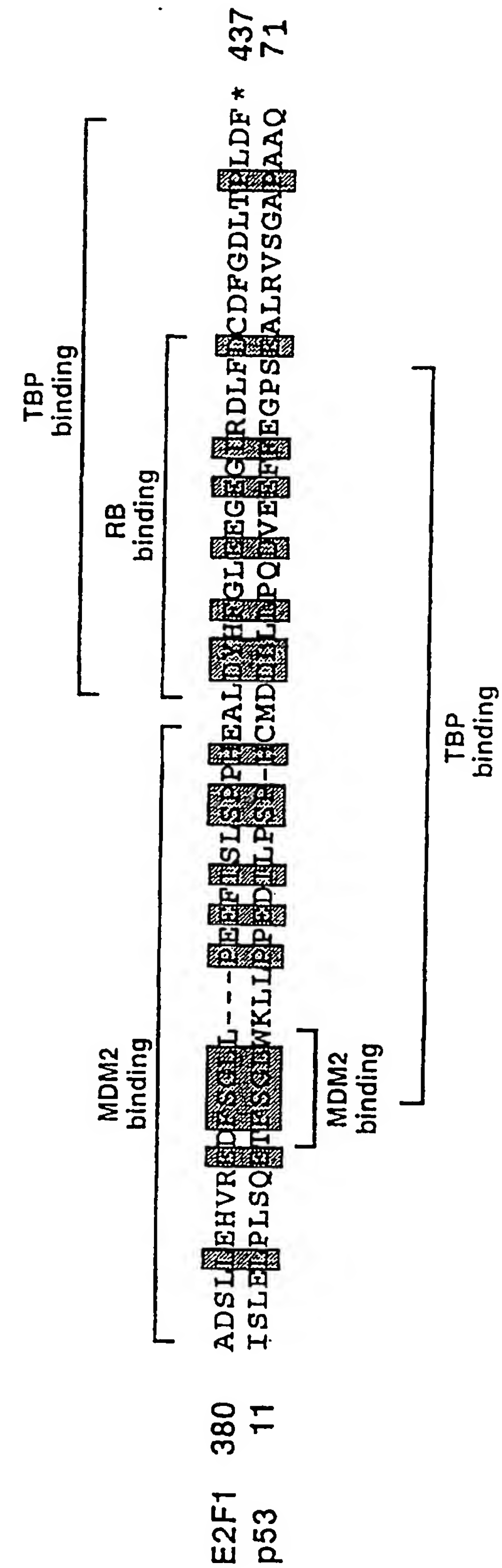

E2F1
p53
380
11
437
71
TBP
binding
RB
binding
MDM2
binding
MDM2
binding
TBP
binding

Figure 3B

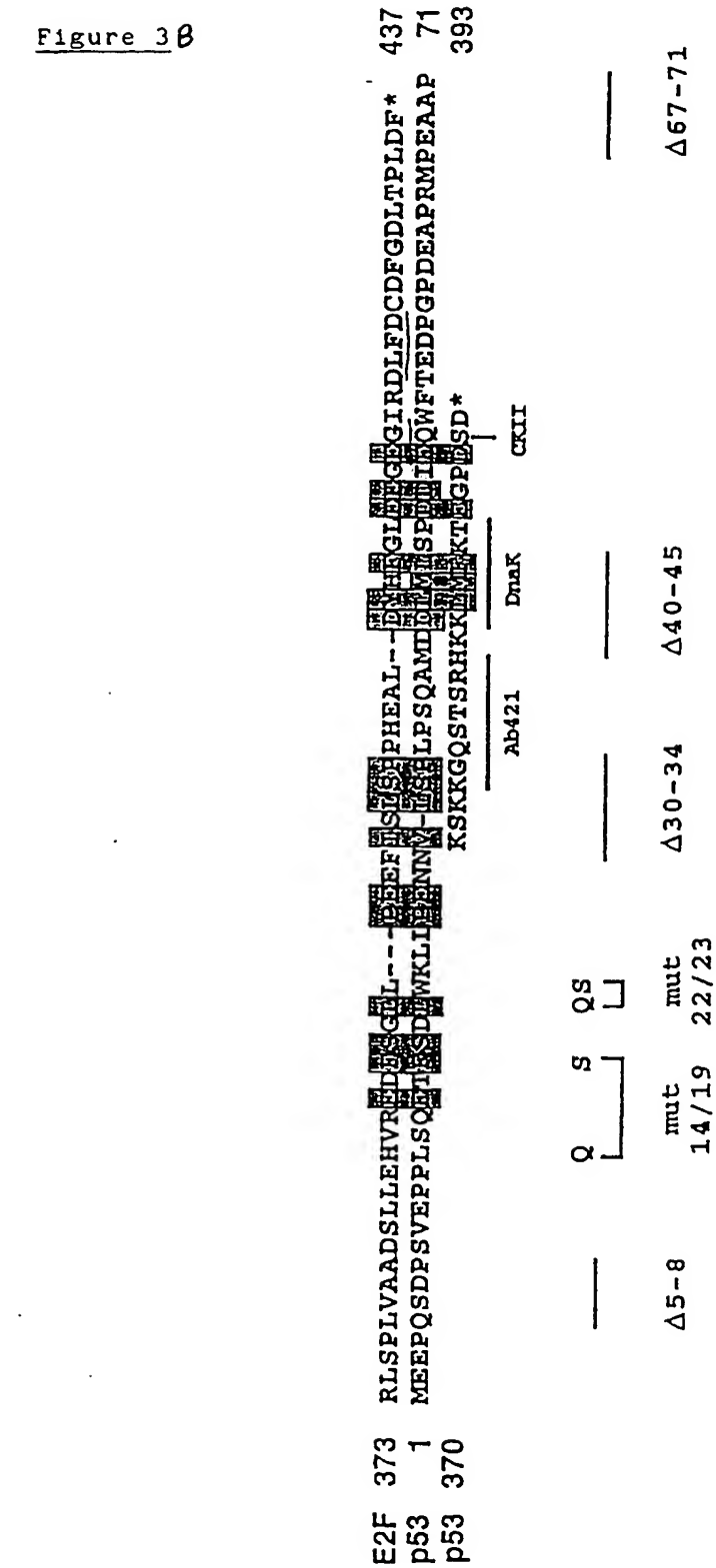
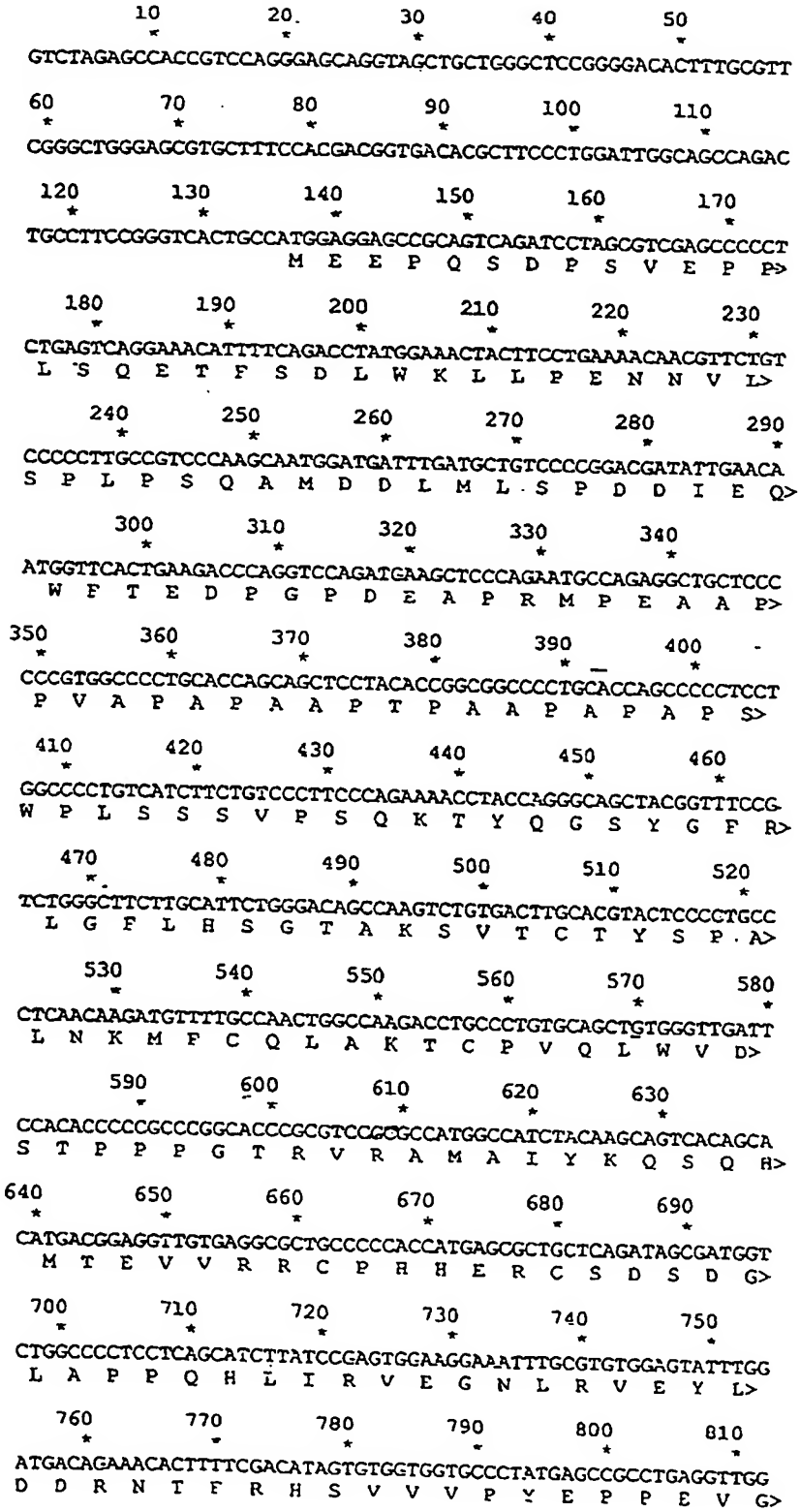

E2F 373
p53 1
p53 370
RLSPLVAADSLLEHVR
MEEPQSDPSVEPPLSQ
KSKKGQSTSRHKK
Ab421
DnaK
CKII
Δ5-8
mut 14/19
Q S
mut 22/23
Δ30-34
Δ40-45
Δ67-71
437
71
393

Figure 3C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60 70 80 90 100 110
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120 130 140 150 160 170
TGCCTTCCGGGTCACTGCCATGGAGGAGCCGCAGTCAGATCCTAGCGTCGAGCCCCCT
M E E P Q S D P S V E P P>
180 190 200 210 220 230
CTGAGTCAGGAAACATTTTCAGACCTATGGAAACTACTTCCTGAAAACAACGTTCTGT
L S Q E T F S D L W K L L P E N N V L>
240 250 260 270 280 290
CCCCCTTGCCGTCCCAAGCAATGGATGATTTGATGCTGTCCCCGGACGATATTGAACA
S P L P S Q A M D D L M L S P D D I E Q>
300 310 320 330 340
ATGGTTCACTGAAGACCCAGGTCCAGATGAAGCTCCCAGAATGCCAGAGGCTGCTCCC
W F T E D P G P D E A P R M P E A A P>
350 360 370 380 390 400
CCCGTGGCCCCTGCACCAGCAGCTCCTACACCGGCGGCCCCTGCACCAGCCCCCTCCT
P V A P A P A A P T P A A P A P A P S>
410 420 430 440 450 460
GGCCCCTGTCATCTTCTGTCCCTTCCCAGAAAACCTACCAGGGCAGCTACGGTTTCCG
W P L S S S V P S Q K T Y Q G S Y G F R>
470 480 490 500 510 520
TCTGGGCTTCTTGCATTCTGGGACAGCCAAGTCTGTGACTTGCACGTACTCCCCTGCC
L G F L H S G T A K S V T C T Y S P A>
530 540 550 560 570 580
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L N K M F C Q L A K T C P V Q L W V D>
590 600 610 620 630
CCACACCCCCGCCCGGCACCCGCGTCCGCGCCATGGCCATCTACAAGCAGTCACAGCA
S T P P P G T R V R A M A I Y K Q S Q H>
640 650 660 670 680 690
CATGACGGAGGTTGTGAGGCGCTGCCCCCACCATGAGCGCTGCTCAGATAGCGATGGT
M T E V V R R C P H H E R C S D S D G>
700 710 720 730 740 750
CTGGCCCCTCCTCAGCATCTTATCCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGG
L A P P Q H L I R V E G N L R V E Y L>
760 770 780 790 800 810
ATGACAGAAACACTTTTCGACATAGTGTGGTGGTGCCCTATGAGCCGCCTGAGGTTGG
D D R N T F R H S V V V P Y E P P E V G>
SUBSTITUTE SHEET (RULE 26)

Figure 3C (Continued)

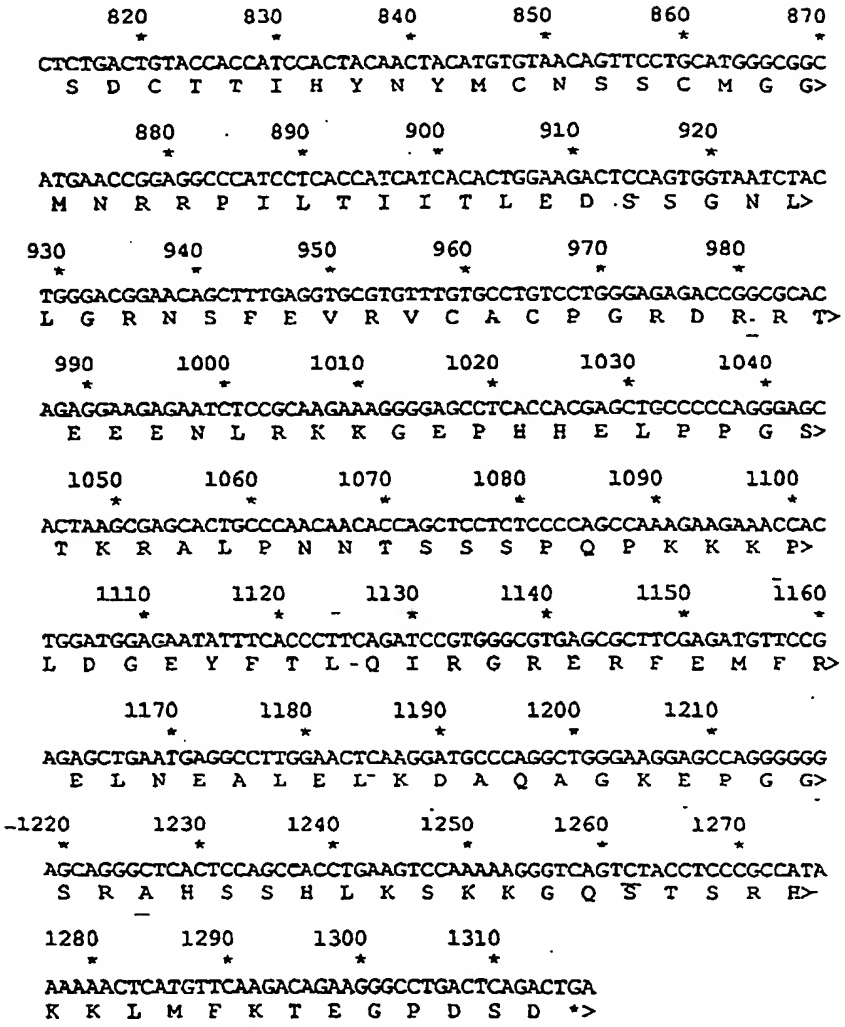


Figure 4

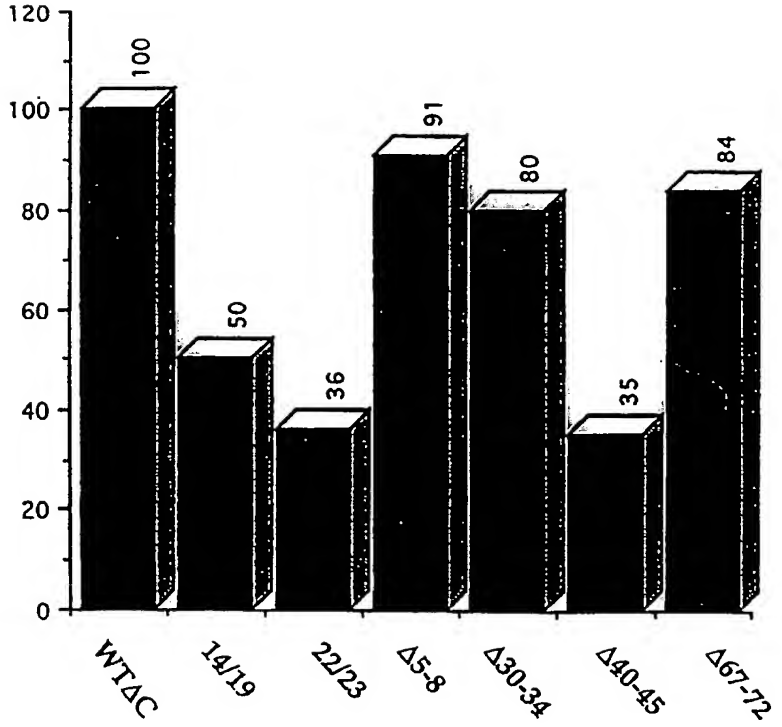

120
100
80
60
40
20
0
100
50
36
91
80
35
84
WTΔC
14/19
22/23
Δ5-8
Δ30-34
Δ40-45
Δ67-72

Figure 5

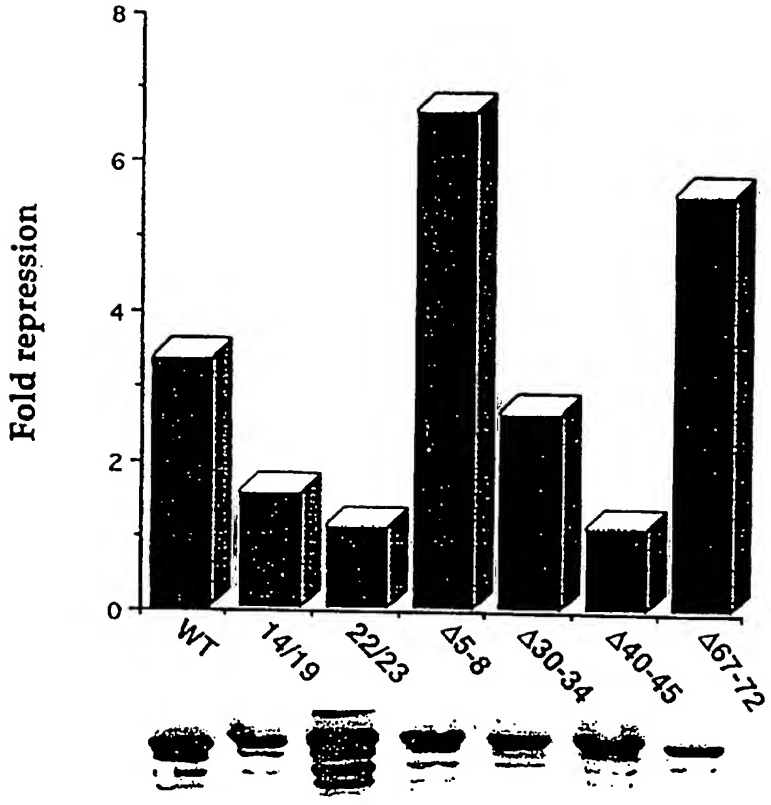


Figure 6

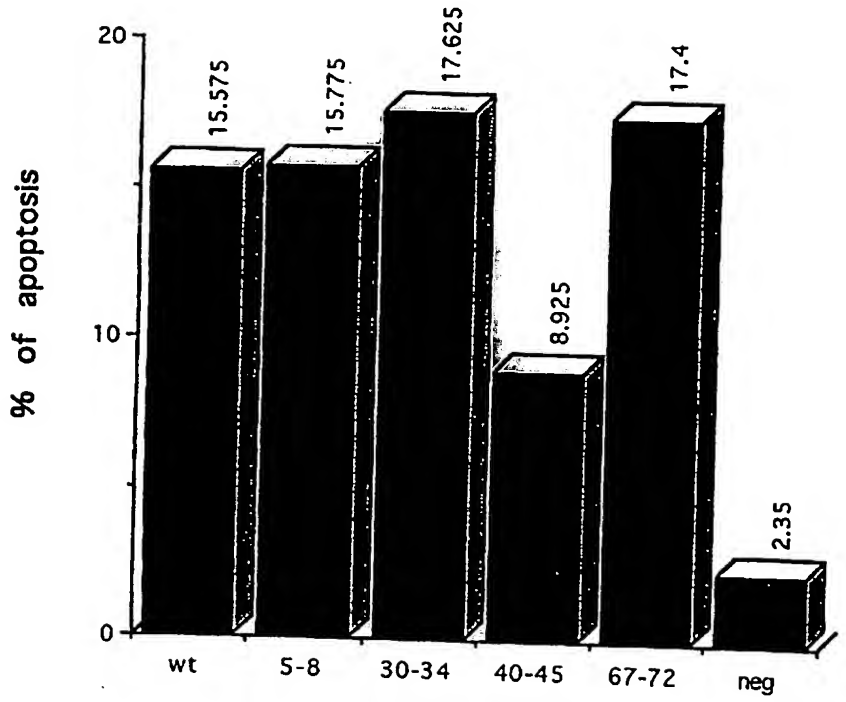
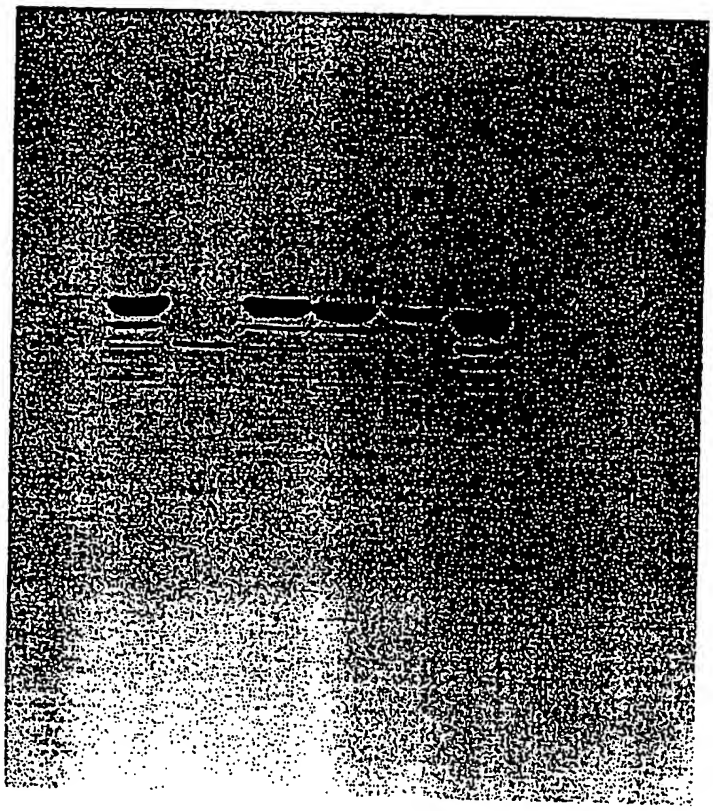

% of apoptosis
20
10
0
15.575
15.775
17.625
8.925
17.4
2.35
wt
5-8
30-34
40-45
67-72
neg

Figure 7



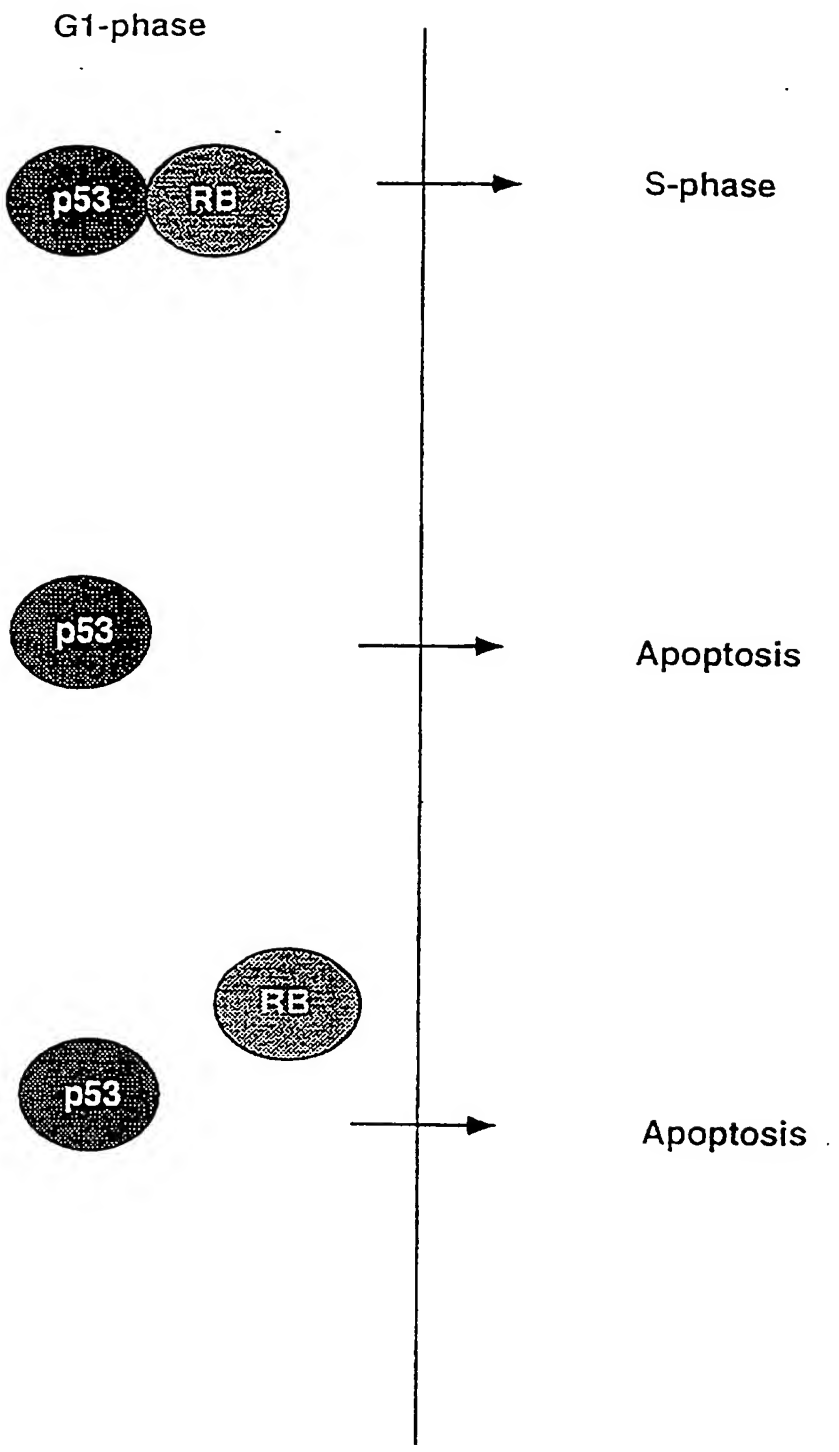


Figure 8.

INTERNATIONAL SEARCH REPORT

Inv tional Application No
PCT/GB 97/01168

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/50 G01N33/53 C07K7/00 C07K14/47 A61K38/03
A61K38/10 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 08241 A (DEUTSCHES KREBSFORSCHUNGSZENTRUM STIFTUNG DES ÖFFENTLICHEN RECHTS) 14 April 1994 see page 19 ---	24-26, 29,30, 33-38
A	TRENDS IN CELL BIOLOGY, vol. 5, no. 12, 1995, pages 448-450, XP002039967 T. KOUZARIDES: "Functions of pRB and p53: what's the conection?" ---	
A	PROC. NATL. ACAD. SCI., vol. 91, April 1994, pages 3602-3606, XP002039816 X. WU ET AL.: "p53 and E2F-1 cooperate to mediate apoptosis." ---	
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
8 September 1997	30-09-1997

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Cartagena y Abella,P

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intr -tional Application No
PCT/GB 97/01168

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THE EMBO JOURNAL , vol. 14, no. 3, 1995, pages 461-472, XP002039817 D. A. HAAS-KOGAN ET AL.: "Inhibition of apoptosis by the retinoblastoma gene product." cited in the application ---	
A	NATURE, vol. 371, 1 September 1994, pages 72-74, XP002039818 S. D. MORGENBESSER ET AL.: "p53-dependent apoptosis produced by Rb-deficiency in the developing mouse lens." cited in the application ---	
P,A	WO 96 20207 A (RESEARCH DEVELOPMENT FOUNDATION) 4 July 1996 ---	
P,A	WO 97 11367 A (CIBA-GEIGY AG) 27 March 1997 -----	

1

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/GB 97/01168

Box I . Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1 [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 53-57, 59
is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos..
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 97/01168

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9408241 A	14-04-94	EP 0614531 A	14-09-94
		JP 7501711 T	23-02-95
WO 9620207 A	04-07-96	AU 4745196 A	19-07-96
WO 9711367 A	27-03-97	AU 7127296 A	09-04-97